Sensitive substrates for neprilysin (neutral endopeptidase) and thermolysin that are highly resistant to serine proteases

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Abstract Tripeptide derivatives like 3-carboxypropanoyl-alanyl-alanyl-leucine 4-nitroanilide or 3-carboxypropanoyl-alanyl-alanyl-phenylalanine 4-nitroanilide are very sensitive substrates for neprilysin ($k_{cat} > 10^2$ s^{-1}; $k_{cat}/K_m \geq 10^6$ s$^{-1}\cdot$M^{-1}) and are widely employed in investigations of the enzyme. However, these compounds are also good substrates for the serine proteases chymotrypsin and subtilisin ($k_{cat} \sim 1$ s^{-1}–34 s^{-1}). By substituting the N-terminal alanine of the substrates with proline, the catalytic efficiency of the enzymic reaction, by the serine proteases, is diminished by 2–3 orders of magnitude, whereas that by neprilysin and thermolysin decreases only slightly. These effects demonstrate that structural alterations in peptide substrates that impair secondary sub-site interactions with one class of peptidases may enhance the selectivity of the substrates towards another class of peptidases.

Key words: Neprilysin; Neutral endopeptidase; CALLA/CD 10; Thermolysin; Serine protease; Substrate selectivity

1. Introduction

The design of highly sensitive and selective substrates for proteolytic enzymes with broad specificities is a difficult task. Neutral endopeptidase (EC 3.4.24.11) or neprilysin (NEP), an ectoenzyme present on the surface of many cell types [1–3], is a zinc metalloendopeptidase with a broad specificity. It hydrolyzes internal peptide bonds of peptides on the amino side of hydrophobic amino acid residues and in this respect it resembles the well-characterized bacterial zinc metalloproteinase thermolysin (EC 3.4.24.27) [4–6]. NEP has been implicated in the metabolism and regulation of a variety of biologically active peptides, e.g. the enkephalins, substance P, atrial natriuretic factor, bradykinin and the chemotactic peptide fMet-Leu-Phe [7,8]. The amino acid sequence of human NEP is identical with that of the common acute lymphoblastic leukemia antigen (CALLA; CD 10) and is highly conserved in different species [9–11].

Most of the highly sensitive synthetic substrates for NEP in current use are amino protected tripeptides linked to a chromogenic or fluorogenic group at the carboxy terminus, with a Phe or Leu at the carboxy terminus serving as the major hydrophobic residue [6,12–14]. These substrates are used in two-stage assays whereby an externally added aminopeptidase releases the chromophore or fluorophore subsequent to enzymatic cleavage of the substrate by NEP. Here we report on the hydrolysis of four pairs of N-blocked tripeptide 4-nitroanilide substrates, with Phe or Leu as the carboxy terminal amino acid, by neprilysin and thermolysin as well as by α-chymotrypsin and subtilisin Carlsberg. We found that a substantial selectivity of the substrates for the metalloendopeptidases versus the serine proteases was gained when Pro was introduced as the N-terminal amino acid residue of the tripeptide derivatives.

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2. Materials and methods

2.1. Substrates

Suc-Ala-Ala-Phe-NH-Np (compound 1) was obtained from Bachem (Budendorf, Switzerland). Suc-Gly-Gly-Phe-NH-Np (compound 7) was obtained from Sigma (St. Louis, MO, USA) or prepared as previously described [15]. The other substrates (compounds 2–6, 8) were synthesized by two-step additions of Ala, Pro or Gly to Phe-NH-Np or Leu-NH-Np, via reaction with the *N*-hydroxysuccinimide esters of the *t*-butoxycarbonyl amino acids and deblocking of the *t*-butoxycarbonyl groups with trifluoroacetic acid, followed by succinylation with succinic anhydride. The products were recrystallized from ethanol/water (compounds 2, 3, 5, 8) or dissolved in ethyl acetate and precipitated with petroleum ether (compounds 4, 6). The newly synthesized compounds were characterized by elemental (C, H, N) analyses and their structures were verified by ^{1}H–^{1}H and ^{1}H–^{13}C two-dimensional NMR techniques [16].

2.2. Enzymes

α-Chymotrypsin (TLCK-treated), subtilisin Carlsberg (protease type VIII) and thermolysin (×3 crystallized, protease type X) were from Sigma. Thermolysin was recrystallized essentially as described [17]. Concentrations of the enzymes were estimated by measuring the absorbance at 280 nm using the following molar absorptivities: 5.00×10^{-4} cm$^{-1}\cdot$M^{-1}, 2.61×10^{-4} cm$^{-1}\cdot$M^{-1}, and 6.11×10^{-4} cm$^{-1}\cdot$M^{-1}, respectively.

Neprilysin was purified from bovine kidney membranes, solubilized with Triton X-100, using a multi-step chromatographic procedure. The chromatographic media and the sequence of their operation were: DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden); concanavalin A-Sepharose (obtained by reacting cyanogen bromide-activated Sepharose 4B, Pharmacia, with Concanavalin A, ×3 crystallized, Biomakor, Rehovot, Israel); Q-sepharose Fast Flow (Pharmacia); Hydroxylapatite (BioRad Laboratories, Richmond, CA, USA); Sephacryl S-200 or S-300 (Pharmacia). Neprilysin concentration was estimated by the Bicinchoninic acid method, using bovine serum albumin as the standard [18]. A polypeptide molecular mass of 85,600, the average mass of rat, rabbit and human neprilysins was used.

2.3. Kinetic measurements

Neprilysin and thermolysin activities were assayed by two-stage assays [12–14]. Reactions were carried out at 30°C in 50 mM Tris-HCl/100 mM NaCl/1 mM $CaCl_2$/0.25 mM Hepes/0.005% Triton X-100, pH 7.5, containing 6.7 μg/ml *Streptomyces griseus* aminopeptidase I [19] when Leu is the C-terminal amino acid of the substrates (compounds 2, 4, 6, 8) or 26.8 μg/ml of the same aminopeptidase when Phe is the C-terminal amino acid (compounds 1, 3, 5, 7). Reactions were performed in 200 μl volumes in 96-well polystyrene microplates (Greiner, Frickenhausen, Germany), using a thermoregulated Thermomax Microplate Reader (Molecular Devices Corp., Menlo Park, CA). The small amount of detergent used, added with the enzyme aliquots (10 μl),

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Table 1
Kinetic parameters for bovine kidney neprilysin acting upon 3-carboxy-propanoyl-tripeptide 4-nitroanilides

No.	Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot M^{-1} \times 10^{-3}$)
	↓			
	$-P_2$ $-P_1$ $-P'_1-P'_2$			
1	Suc-Ala-Ala-Phe-NH-Np	131.5 ± 2.8	0.076 ± 0.005	1730 ± 70
2	Suc-Ala-Ala-Leu-NH-Np	306.0 ± 13.7	0.290 ± 0.014	1058 ± 9
3	Suc-Pro-Ala-Phe-NH-Np	54.3 ± 2.2	0.050 ± 0.003	1082 ± 22
4	Suc-Pro-Ala-Leu-NH-Np	199.5 ± 1.1	0.638 ± 0.053	314.7 ± 26.7
5	Suc-Ala-Pro-Phe-NH-Np	13.41 ± 0.49	0.026 ± 0.003	519.9 ± 50.2
6	Suc-Ala-Pro-Leu-NH-Np	88.5 ± 4.9	0.374 ± 0.042	237.6 ± 13.0
7	Suc-Gly-Gly-Phe-NH-Np	0.89 ± 0.014	0.013 ± 0.0008	67.1 ± 4.2
8	Suc-Gly-Gly-Leu-NH-Np	3.17 ± 0.032	0.094 ± 0.0045	34.0 ± 1.3

Reactions were carried out at pH 7.5, 30°C. Enzyme concentrations were 18.1 ng/ml–1.81 μg/ml, depending on the substrate. Substrate concentration range was 0.05 mM–2 mM.

was introduced in order to minimize enzyme adsorption to the microplates. α-Chymotrypsin and subtilisin Carlsberg activities were assayed at 25°C in 50 mM Tris-HCl/100 mM NaCl/1 mM $CaCl_2$, pH 7.5 in 1 ml quartz cuvettes, using a Varian Techtron Model 635 spectrophotometer. Activities of all the enzymes were measured by following the increase in absorbance at 405 nm, due to the release of 4-nitroaniline, $\varepsilon_{405} = 10{,}600\ cm^{-1} \cdot M^{-1}$ [20]. Values of k_{cat} and K_m were derived by the Lineweaver–Burk plots. Each data point is the average of three to four assays. The kinetic measurements of neprilysin and thermolysin were carried out in three separate experiments and the kinetic parameters are the mean ± S.D.

3. Results and discussion

The pair of substrates Suc-Ala-Ala-Phe-NH-Np and Suc-Ala-Ala-Leu-NH-Np (compounds 1 and 2) are very sensitive substrates for neprilysin with k_{cat} values of the order of $10^2\ s^{-1}$ and k_{cat}/K_m values of the order $10^6\ s^{-1} \cdot M^{-1}$ (Table 1). These substrates are hydrolyzed at the Ala–Phe and Ala–Leu bonds, respectively, conforming with the primary specificity of the enzyme, known to cleave internal peptide bonds of peptides on the amino side of hydrophobic amino acid residues [1,6,13]. The hydrophobic Phe or Leu residues of the substrates interact with the primary specificity site of the enzyme S'_1, whereas the two Ala residues interact with the secondary sub-sites S_1 and S_2 (according to the nomenclature of Schechter and Berger; [21]). Even the 4-nitroanilide group possibly interacts with a sub-site, S'_2, on the surface of the enzyme. Compounds 1 and 2 are two orders of magnitude more sensitive substrates than compounds 7 and 8, having Gly residues instead of the Ala residues at P_1 and P_2, thereby demonstrating the importance of the side chains in the secondary sub-site interactions of peptide substrates with the enzyme (Table 1). Substitution of the N-terminal Ala residue (at P_2) with Pro (compounds 3, 4) has only a slight effect on the sensitivity of the substrates whereas substitution of the Ala residue at P_1 with Pro (compounds 5, 6) has a more pronounced effect in diminishing the activity (Table 1).

The pattern of activities of thermolysin towards the same series of substrates is similar to that of neprilysin, although the absolute values of the kinetic parameters are not identical for the two enzymes (Tables 1 and 2). It is known that neprilysin and thermolysin share basic elements of specificity and mechanistic properties [1,4,5,8], and the present results reflect upon the similarities (and differences) between the enzymes in a region encompassing 3–4 sub-sites, including the primary specificity site of the enzyme, S'_1. Phe at position P'_1 yields lower K_m compared with Leu at the same position, similarly to that observed for neprilysin (compounds 1, 3, 5, 7 versus compounds 2, 4, 6, 8, respectively; Tables 1 and 2). Substitution of the two Ala residues at P_1 and P_2 with Gly residues (compounds 7, 8 versus compounds 1, 2; Table 2) diminish the sensitivity by more than two orders of magnitude. Substitution of the N-terminal Ala at P_2 with Pro (compounds 3, 4) has only a slight effect on the sensitivity, but substitution of Ala at P_1 with Pro (compounds 5, 6) has a much more pronounced effect in diminishing the activity (Tables 1 and 2).

When compounds 1 and 2 are acted upon by the serine proteases α-chymotrypsin and subtilisin Carlsberg, hydrolysis occurs on the carboxyl side of the Phe or Leu residue which now occupy the primary specificity site S_1 of these enzymes [22,23]. Subsequently the two Ala residues occupy subsites S_2 and S_3 of these enzymes and the 4-nitroanilide group possibly

Table 2
Kinetic parameters for thermolysin acting upon 3-carboxypropanoyl-tripeptide 4-nitroanilides

No.	Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot M^{-1} \times 10^{-3}$)
	↓			
	$-P_2$ $-P_1$ $-P'_1-P'_2$			
1	Suc-Ala-Ala-Phe-NH-Np	152.4 ± 9.3	0.589 ± 0.046	259.4 ± 5.1
2	Suc-Ala-Ala-Leu-NH-Np	1786.7 ± 99.7	7.45 ± 0.38	239.8 ± 9.3
3	Suc-Pro-Ala-Phe-NH-Np	68.3 ± 2.7	0.521 ± 0.035	131.0 ± 3.5
4	Suc-Pro-Ala-Leu-NH-Np	610.8 ± 33.9	5.75 ± 0.41	106.4 ± 2.1
5	Suc-Ala-Pro-Phe-NH-Np	6.20 ± 0.17	1.23 ± 0.04	5.04 ± 0.11
6	Suc-Ala-Pro-Leu-NH-Np	72.3 ± 0.8	8.95 ± 0.14	8.09 ± 0.047
7	Suc-Gly-Gly-Phe-NH-Np	0.28 ± 0.005	0.286 ± 0.014	0.97 ± 0.030
8	Suc-Gly-Gly-Leu-NH-Np	5.85 ± 0.36	4.95 ± 0.43	1.18 ± 0.028

Reactions were carried out at pH 7.5, 30°C. Enzyme concentrations were 3.4 ng/ml–4.9 μg/ml, depending on the substrate. Substrate concentration range was 0.05 mM–2 mM for the Phe-containing substrates (compounds 1, 3, 5, 7), 0.4 mM–8 mM for compounds 2 and 8, 0.4 mM–7 mM for compounds 4 and 6.

Table 3
Kinetic parameters for α-chymotrypsin and subtilisin Carlsberg acting upon 3-carboxypropanoyl-tripeptide 4-nitroanilides

No.	Substrate	α-Chymotrypsin			Subtilisin Carlsberg		
		k_{cat} s^{-1}	K_m mM	k_{cat}/K_m $s^{-1} \cdot M^{-1} \times 10^{-3}$	k_{cat} s^{-1}	K_m mM	k_{cat}/K_m $s^{-1} \cdot M^{-1} \times 10^{-3}$
	↓ $-P_3$ $-P_2$ $-P_1$ $-P'_1$						
1	Suc-Ala-Ala-Phe-NH-Np	8.41	0.097	86.7	19.46	0.49	39.7
2	Suc-Ala-Ala-Leu-NH-Np	1.11	0.77	1.44	33.94	0.93	36.5
3	Suc-Pro-Ala-Phe-NH-Np	0.20	1.16	0.17	0.113	0.89	0.13
4	Suc-Pro-Ala-Leu-NH-Np	0.0063	1.48	0.0043	0.62	3.68	0.17
5	Suc-Ala-Pro-Phe-NH-Np	10.22	0.039	262	3.46	0.74	4.68
6	Suc-Ala-Pro-Leu-NH-Np	1.70	0.36	4.72	44.62	13.49	3.31
7	Suc-Gly-Gly-Phe-NH-Np	1.13	0.84	1.35	0.93	0.69	1.34
8	Suc-Gly-Gly-Leu-NH-Np	0.017	1.38	0.012	2.75	3.73	0.74

Reactions were carried out at pH 7.5, 25°C. α-Chymotrypsin concentration was 0.45 μg/ml–49.5 μg/ml and subtilisin Carlsberg concentration 0.36 μg/ml–18.6 μg/ml, depending on the substrate. Substrate concentration range was 0.05 mM–1 mM for compounds 1 and 5, 0.1 mM–4 mM for compounds 2 and 8, 0.1 mM–2 mM for compound 3, 0.2 mM–4 mM for compounds 4, 6 and 7.

interacts with sub-site S'_1. Compound 1 with Phe at position P_1 is hydrolyzed at a much faster rate than compound 2, with Leu at the same position, when α-chymotrypsin is the protease, but apparently the two substrates behave similarly when subtilisin Carlsberg is the hydrolyzing enzyme (Table 3). Again, substitution of the two Ala residues at P_2 and P_3 with Gly (compounds 7, 8 versus compounds 1, 2) abolish the catalytic efficiency of the enzymic reaction by two orders of magnitude for both enzymes (Table 3). Thus, in all four proteases/peptidases here presented the substitution of two Ala residues with Gly residues, that interact with secondary sub-sites of the enzymes, abolishes k_{cat} and/or k_{cat}/K_m to a comparable extent (Tables 1–3). The most remarkable effect on substrate selectivity in this study is seen in the substitution of the N-terminal Ala with Pro (compounds 3, 4 versus compounds 1, 2). The catalytic efficiency is only slightly decreased when the zinc metallopeptidases neprilysin and thermolysin are considered (Tables 1, 2, 4)., but it is abolished by 2–3 orders of magnitude when the serine proteases α-chymotrypsin and subtilisin Carlsberg are employed (Tables 3 and 4).

Early X-ray crystallographic studies of α-chymotrypsin [24] and subtilisin BPN′ [25] with irreversible chloromethylketone extended peptide inhibitors suggest that extended peptide substrates bind to the active site of these enzymes as in an antiparallel β-pleated sheet. In this proposal, two hydrogen bonds are contributed by the backbone NH and CO groups of the substrate residue at position P_3 and one hydrogen bond is contributed by the backbone NH of the substrate residue at position P_1 [24,25]. No hydrogen bonding with the enzyme is assigned to the substrate residue at P_2. The structure of the complex formed between subtilisin Carlsberg and eglin c seems to suggest a similar mode of binding of an extended peptide substrate to subtilisin Carlsberg [26]. Elimination of one of these interactions upon substitution of Ala at P_3 with Pro (compounds 3, 4 versus compounds 1, 2) could possibly account for the loss of activity by the serine proteases, although steric restrictions resulting from this substitution may also contribute [24]. More recent X-ray crystallographic investigations of the complexes formed between thermolysin and its inhibitors also led to a proposal as to the mode of binding of extended peptide substrates to thermolysin [27,28]. Again, the substrate is bound to the enzyme as in an antiparallel β-pleated sheet, with two hydrogen bonds contributed by the backbone of the substrate residue at position P_2 and one hydrogen bond by the NH group of the residue at position P'_1. However, additional anchorage points of the substrate to the enzyme are assigned to the NH and CO groups of the residue at position P_1, with the phenolic oxygen of Tyr-157 and the active site zinc, respectively [28]. Apparently, the release of one of these interactions upon substitution of the N-terminal Ala with Pro (compounds 3, 4 versus compounds 1, 2) is of a much smaller effect in thermolysin compared with its effect in the serine proteases (Table 4). Determination of the three-dimensional structure of neprilysin and its complexes with inhibitors should allow exploration of the

Table 4
Catalytic efficiency ratios of neprilysin, thermolysin, α-chymotrypsin and subtilisin Carlsberg acting upon 3-carboxypropanoyl-tripeptide 4-nitroanilides[a]

No.	Substrate	k_{cat}/K_m ratios			
		Neprilysin[b]	Thermolysin[c]	α-Chymotrypsin[d]	Subtilisin Carlsberg[d]
1	Suc-Ala-Ala-Phe-NH-Np	1.00	1.00	1.00	1.00
2	Suc-Ala-Ala-Leu-NH-Np	0.61	0.92	0.017	0.92
3	Suc-Pro-Ala-Phe-NH-Np	0.63	0.51	0.0020	0.0032
4	Suc-Pro-Ala-Leu-NH-Np	0.18	0.41	0.000050	0.0043

[a]Activity towards Suc-Ala-Ala-Phe-NH-Np for all the enzymes is taken as 1.00.
[b]At 30°C, calculated from data of Table 1.
[c]At 30°C, calculated from data of Table 2.
[d]At 25°C, calculated from data of Table 3.

possible interactions between the substrate's backbone and the enzyme and their resemblance to those prevailing in thermolysin.

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